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<p>(21) International Application Number: PCT/US97/03994</p> <p>(22) International Filing Date: 3 March 1997 (03.03.97)</p> <p>(71) Applicant (for all designated States except US): MONSANTO COMPANY [US/US]; 800 North Lindbergh Boulevard, St. Louis, MO 63167 (US).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): HEIN, Silke [DE/DE]; Rosenplatz 1a, D-48143 Münster (DE). SÖHLING, Brigitte [DE/DE]; Hochstrasse 2, D-06110 Halle (DE). GOTTSCHALK, Gerhard [DE/DE]; Johann-Wolfe-Strasse 35a, D-37176 Nörten-Hardenberg (DE). STEINBÜCHEL, Alexander [DE/DE]; Rönneithal 27, D-48341 Altenberge (DE).</p> <p>(74) Agent: KAMMERER, Patricia, A.; Arnold, White &amp; Durkee, P.O. Box 4433, Houston, TX 77210 (US).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> With international search report.</p>
<p>(54) Title: METHODS FOR THE BIOSYNTHESIS OF POLYESTERS</p> <p>(57) Abstract</p> <p>Coexpression of a polyhydroxyalkanoic acid synthase and either a fatty acid: acyl-CoA transferase or an acyl-CoA synthetase in cells enables the biosynthesis of polyester materials. Plasmids, bacteria, materials, and methods for the preparation of polyesters are disclosed.</p>		

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## METHODS FOR THE BIOSYNTHESIS OF POLYESTERS

### FIELD OF THE INVENTION

The invention relates generally to the field of molecular biology. Certain  
5   embodiments entail materials and methods suitable for the biosynthesis of biopolymers,  
namely polyhydroxyalkanoic acid polymers.

### BACKGROUND OF THE INVENTION

The production of intracellular polyesters belonging to the class of polymers known as  
polyhydroxyalkanoates (polyhydroxyalkanoic acids) has been observed in a wide array of  
10   prokaryotic organisms (Anderson, A.J. and Dawes, E.A. (1990) *Microbiol. Rev.* 54:450-472;  
Steinbüchel, A. and Valentin, H.E. (1995) *FEMS Microbiol. Lett.* 128:219-228). The  
monomers composing the polyesters range in length from C4 (3-hydroxybutyrate) to C12 (3-  
hydroxydodecanoate) (Lageveen, R.G. et al. (1988) *Appl. Env. Microbiol.* 54:2924-2932).  
These polyesters have attracted considerable interest as they are biodegradable. Potential  
15   technical applications exist in industry and agriculture, as well as in medical devices and  
procedures (Hocking, P.J. and Marchessault, R.H. (1994) Biopolyesters. In: G.J.L. Griffin  
(Ed) Chemistry and technology of biodegradable polymers, Chapman & Hall, London, pp.48-  
96; Müller, H.M. and Seebach, D. (1993) *Angew. Chem.* 105:483-509). Additionally, this  
class of polyesters is attractive as a potential alternative to conventional petrochemical-derived  
20   plastics.

Polyhydroxyalkanoic acids are broadly characterized according to the monomers that  
constitute their backbone. Polymers composed of C4-C5 units are classified as short chain  
length (scl) polyhydroxyalkanoic acids; polymers containing monomers of C6 units and above  
are classified as medium chain length (mcl) polyhydroxyalkanoic acids. The primary  
25   structure of the polymer influences the physical properties of the polyester.

The metabolic pathways leading to the formation of polyhydroxyalkanoic acids have  
not been elucidated for all organisms. The most extensively studied polyhydroxyalkanoic  
acid biosynthetic pathway is that of *Alcaligenes* (Peoples, O.P. et al. (1989) *J. Biol. Chem.*

264:15298-15303; Valentin, H.E. et al. (1995) *Eur. J. Biochem.* 227:43-60). This organism is capable of forming either a homopolymer of C4 (polyhydroxybutyrate, PHB) or a co-polymer of C4-C5 (PHB-PHV, polyhydroxybutyrate-polyhydroxyvalerate) (Koyama, N. and Doi, Y. (1995) *Biotechnol. Lett.* 17:281-284). Hence, *A. eutrophus* is classified as a scl

5 polyhydroxyalkanoic acid organism. Similarly, *Pseudomonas* species generate a polymer composed of monomers ranging in length from C6 to C12 (Timm, A. and Steinbüchel, A. (1990) *Appl. Environ. Microbiol.* 56:3360; Lageveen, R.G. et al. (1988) *Appl. Env. Microbiol.* 54:2924-2932), and are classified as mcl polyhydroxyalkanoic acid organisms.

The polymerization of the hydroxyacyl-CoA substrates is carried out by

10 polyhydroxyalkanoic acid synthases. The substrate specificity of this class of enzyme varies across the spectrum of polyhydroxyalkanoic acid producing organisms. This variation in substrate specificity of polyhydroxyalkanoic acid synthases is supported by indirect evidence observed in heterologous expression studies (Lee, E.Y. et al. (1995) *Appl. Microbiol. Biotechnol.* 42:901-909; Timm, A. et al. (1990) *Appl. Microbiol. Biotechnol.* 33:296-301).

15 Hence, the structure of the backbone of the polymer is strongly influenced by the polyhydroxyalkanoic acid synthase responsible for its formation.

Fluorescent pseudomonads belonging to the rRNA homology group I can synthesize and accumulate large amounts of polyhydroxyalkanoic acids (PHA) composed of various saturated and unsaturated hydroxy fatty acids with carbon chain lengths ranging from 6 to 14

20 carbon atoms (Steinbüchel, A. and Valentin, H.E. (1992) *FEMS Microbiol. Rev.* 103:217). Polyhydroxyalkanoic acid isolated from these bacteria also contains constituents with functional groups such as branched, halogenated, aromatic or nitrile side-chains (Steinbüchel and Valentin (1995) *FEMS Microbiol. Lett.* 128:219-228). The composition of polyhydroxyalkanoic acid depends on the polyhydroxyalkanoic acid polymerase system, the

25 carbon source, and the metabolic routes (Anderson, A.J. and Dawes, E.A. (1990) *Microbiol. Rev.* 54:450-472; Eggink et al. (1992) *FEMS Microbiol. Rev.* 105:759; Huisman, A.M. et al. (1989) *Appl. Microbiol. Biotechnol.* 55:1949-1954; Lenz, O. et al. (1992) *J. Bacteriol.* 176:4385-4393; Steinbüchel, A. and Valentin, H.E. (1995) *FEMS Microbiol. Lett.* 128:219-228). In *P. putida*, at least three different metabolic routes occur for the synthesis of 3-

hydroxyacyl CoA thioesters, which are the substrates of the polyhydroxyalkanoic acid synthase (Huijberts, G.N.M. et al. (1994) *J. Bacteriol.* 176:1661-1666): (i)  $\beta$ -oxidation is the main pathway when fatty acids are used as carbon source; (ii) *De novo* fatty acid biosynthesis is the main route during growth on carbon sources which are metabolized to acetyl-CoA, like gluconate, acetate or ethanol; and (iii) Chain elongation reaction, in which acyl-CoA is condensed with acetyl-CoA to the two carbon chain extended  $\beta$ -keto product which is then reduced to 3-hydroxyacyl-CoA. This latter pathway is involved in polyhydroxyalkanoic acid-synthesis during growth on hexanoate.

The polyhydroxyalkanoic acid synthase structural gene from *Alcaligenes eutrophus* (*phaC<sub>Ae</sub>*) has been cloned and characterized at the molecular level in several laboratories (for a review see Steinbüchel, A. and Schlegel, H.G. (1991) *Mol. Microbiol.* 5:535-542; GenBank Accession number J05003). It was demonstrated that *phaC<sub>Ae</sub>* in combination with other genes conferred the ability to synthesize poly(3-hydroxybutyric acid) not only to many bacteria, which do not synthesize this polyester such as e.g. *Escherichia coli* (Steinbüchel, A. and Schlegel, H.G. (1991) *Mol. Microbiol.* 5:535-542) but also to *Saccharomyces cerevisiae* (Leaf, T.A. et al. (1996) *Microbiology* 142:1169-1180), plants such as *Arabidopsis thaliana* (Poirier, Y. et al. (1992) *Science* 256:520-523.) and *Gossypium hirsutum* (John, M.E. and Keller, G. (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93:12768-12773), and even to cells from the insect *Spodoptera frugiperda* (Williams, M.D. et al. (1996) *Appl. Environ. Microbiol.* 62:2540-2546).

The development of biological systems that synthesize poly (4-hydroxybutyric acid), poly (3-hydroxybutyric acid -co- 4-hydroxybutyric acid), and other polyester materials would be of great utility. Biological systems provide the potential to produce significant quantities of important materials, while utilizing inexpensive feedstocks and minimizing hazardous byproducts.

There exists a need for novel biosynthetic routes to polymers of potential commercial interest that do not rely on petroleum based starting materials. Biological processes present an

attractive alternative to chemical processes that produce potentially harmful byproducts while consuming non-renewable resources.

### **SUMMARY**

This invention relates to materials and processes for preparing polyester materials.

5 More particularly, the invention is related to materials and processes for the preparation of polyester materials, preferably poly (4-hydroxybutyric acid), poly (3-hydroxybutyric acid), and poly (3-hydroxybutyric acid -co- 4-hydroxybutyric acid). The invention provides nucleic acid segments encoding a polyhydroxyalkanoic acid synthase protein and either a fatty acid:acyl-CoA transferase protein or an acyl-CoA synthetase protein, recombinant vectors,  
10 cells containing the nucleic acid segments, and methods for the preparation of polyester materials.

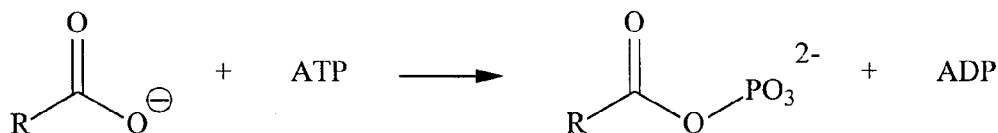
The scope of the present invention will be further apparent in light of the detailed descriptions provided below. However, it should be understood that the following detailed description and examples, while indicating preferred embodiments of the present invention,  
15 are given by way of illustration only since various changes and modifications within the spirit and scope of the present invention will become apparent to those of ordinary skill in the art from this detailed description.

### **Definitions**

The following definitions are provided in order to aid those skilled in the art in  
20 understanding the detailed description of the present invention.

An “acyl-CoA synthetase” or “thiokinase” protein catalyzes the formation of a thioester linkage between the carboxyl group of a fatty acid and the sulfhydryl group of CoA.

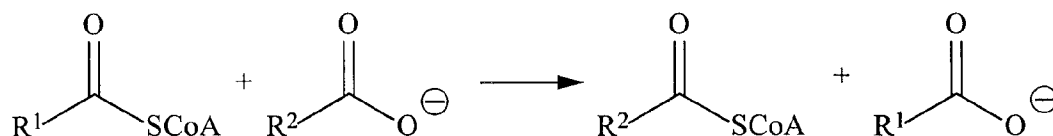
An “acyl kinase” protein catalyzes the transfer of a phosphate group from ATP to a carboxylate group according to the reaction:



wherein R is an alkyl or hydroxyalkyl group.

“CoA” refers to coenzyme A.

The term “fatty acid:acyl-Co A transferase” refers to a protein that catalyzes an acyl group transfer according to the reaction:



- 5 wherein R<sup>1</sup> and R<sup>2</sup> are alkyl or hydroxyalkyl groups. Groups R<sup>1</sup> and R<sup>2</sup> may further contain one or more double bonds, triple bonds, or aromatic groups.

- “Copolyester” refers to a polyester material made from a two different monomeric building blocks. For example, poly (3-hydroxybutyric acid -co- 4-hydroxybutyric acid) is a polyester made from 3-hydroxybutyric acid and 4-hydroxybutyric acid. The relative composition of the two monomeric building blocks in the copolyester can be variable. Copolyesters are commonly characterized by the relative percentages of the two monomeric building blocks. The percentage composition may affect the physical characteristics of the copolyester.
- 10

- “Dimeric” refers to enzymes that are comprised of two protein molecule subunits. The two subunits may be identical (homodimeric) or different (heterodimeric) in sequence.
- 15

“Heterologous” refers to nucleotide segments not normally found in nature in the same organism.

- “Homopolyester” refers to a polyester material made from a single monomeric building block. For example, poly (4-hydroxybutyric acid) is a polyester made from 4-hydroxybutyric acid.
- 20

A “4-hydroxybutyrate dehydrogenase” protein catalyzes the conversion of succinate semialdehyde to 4-hydroxybutyrate.

The combination of “2-methylcitrate dehydratase” protein and “2-methylisocitrate dehydratase” protein catalyzes the conversion of 2-methylcitrate to 2-methylisocitrate.

- 5           A “2-methylcitrate synthase” protein catalyzes the conversion of propionyl-CoA and oxaloacetate to 2-methylcitrate.

A “2-methylisocitrate lyase” protein catalyzes the conversion of 2-methylisocitrate to pyruvate and succinate.

- 10           The terms “microbe”, “microorganism”, and “microbial” refer to algae, bacteria, fungi, and protozoa.

“Monomeric” refers to enzymes that are comprised of a single protein molecule.

“Native” refers to two segments of nucleic acid naturally occurring in the same organism. For example, a native promoter is the promoter naturally found with a given gene in an organism.

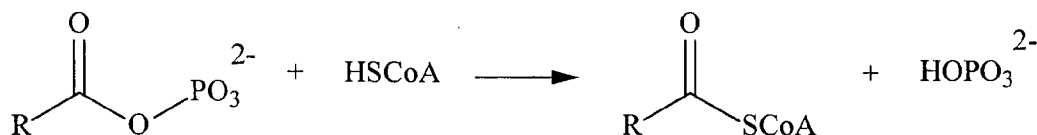
- 15           “Nucleic acid” refers to deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).

- “Overexpression” refers to the expression of a polypeptide or protein encoded by a DNA introduced into a host cell, wherein said polypeptide or protein is either not normally present in the host cell, or wherein said polypeptide or protein is present in said host cell at a higher level than that normally expressed from the endogenous gene encoding said  
20   polypeptide or protein.



A “2-oxoglutarate decarboxylase” protein catalyzes the conversion of 2-oxoglutarate to succinate semialdehyde.

A “phosphotransacylase” protein catalyzes the transfer of a phosphorylated acyl group to CoA according to the reaction:



- 5        The phrases “polyhydroxyalkanoic acid biosynthetic genes” and “polyhydroxyalkanoic acid biosynthetic enzymes” refer to those genes or enzymes leading to anabolic reactions in the pathway of polyhydroxyalkanoic acid production.

The phrase “polyhydroxyalkanoate (PHA) synthase” refers to enzymes that convert hydroxyacyl-CoAs to polyhydroxyalkanoates and free CoA.

- 10        The term “promoter” or “promoter functional in bacteria” refer to a nucleotide sequence, usually found upstream (5') to a coding sequence, that controls expression of the coding sequence by controlling production of messenger RNA (mRNA) by providing the recognition site for RNA polymerase and/or other factors necessary for the start of transcription at the correct site. The promoters disclosed herein, and biologically functional
- 15        equivalents thereof, are responsible for driving the transcription of coding sequences under their control when introduced into a bacterial cell, as demonstrated by their ability to produce mRNA.

- 20        The terms “recombinant vector” and “vector” refer to any agent such as a plasmid, cosmid, virus, autonomously replicating sequence, phage, or linear or circular single-stranded or double-stranded DNA or RNA nucleotide sequence, derived from any source, capable of genomic integration or autonomous replication, comprising a DNA molecule in which one or more DNA sequences have been linked in a functionally operative manner (“operatively linked”). Such recombinant DNA constructs or vectors are capable of introducing a 5'

regulatory sequence or promoter region and a DNA sequence for a selected gene product into a cell in such a manner that the DNA sequence is transcribed into a functional mRNA which is translated and therefore expressed.

5           A “succinate-semialdehyde dehydrogenase” protein catalyzes the conversion of succinyl-CoA to succinate semialdehyde.

A “succinate:acetyl-CoA transferase” protein catalyzes the conversion of succinate to succinyl-CoA.

### **DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS**

10           The present invention provides a novel method for the preparation of polyester materials. In one important embodiment, co-expression of a polyhydroxyalkanoic acid synthase gene and a fatty acid:acyl-CoA transferase gene in a cell enable the biosynthesis of poly (4-hydroxybutyric acid). In an alternative embodiment, the co-expression of a polyhydroxyalkanoic acid synthase gene and a fatty acid:acyl-CoA transferase gene in  
15 bacteria leads to the biosynthesis of poly (3-hydroxybutyric acid). In a further alternative embodiment, co-expression of a polyhydroxyalkanoic acid synthase gene and a fatty acid:acyl-CoA transferase gene in a cell enable the biosynthesis of the copolyester poly (3-hydroxybutyric acid -co- 4-hydroxybutyric acid). In an alternative embodiment, co-expression of a polyhydroxyalkanoic acid synthase gene and a fatty acid:acyl-CoA transferase  
20 gene in a cell enable the biosynthesis of poly (4-hydroxybutyric acid).

Alternatively, the co-expression of a polyhydroxyalkanoic acid synthase gene and an acyl-CoA synthetase gene in a cell leads to the biosynthesis of poly (3-hydroxybutyric acid). In a further alternative embodiment, co-expression of a polyhydroxyalkanoic acid synthase gene and an acyl-CoA synthetase gene in a cell enable the biosynthesis of the copolyester  
25 poly (3-hydroxybutyric acid -co- 4-hydroxybutyric acid). In an alternative embodiment, co-expression of a polyhydroxyalkanoic acid synthase gene and an acyl-CoA synthetase gene in a cell enable the biosynthesis of poly (4-hydroxybutyric acid).

In one important embodiment, the invention provides a nucleic acid segment that encodes a polyhydroxyalkanoic acid synthase protein, and that encodes a fatty acid:acyl-CoA transferase protein. The polyhydroxyalkanoic acid synthase protein may generally be any polyhydroxyalkanoic acid synthase protein suitable for the production of polyester materials according to the inventive processes. The polyhydroxyalkanoic acid synthase protein may be monomeric or dimeric. Monomeric polyhydroxyalkanoic acid synthase proteins include, but are not limited to, those from *Rhizobium meliloti*, *Alcaligenes eutrophus*, *Alcaligenes sp.*, *Rhizobium etli*, *Paracoccus denitrificans*, *Acinetobacter sp.*, *Rhodobacter sphaeroides*, *Methylobacterium extorquens*, *Pseudomonas oleovorans*, *Pseudomonas aeruginosa*, *Rhodococcus ruber*, and *Zoogloea ramigera*. Dimeric polyhydroxyalkanoic acid synthase proteins include, but are not limited to, those from *Thiocystis violacea* and *Chromatium vinosum*. Preferably, the polyhydroxyalkanoic acid synthase protein is an *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein, and more preferably, the *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein encoded by the *Alcaligenes eutrophus phaC* polyhydroxyalkanoic acid synthase structural gene. The fatty acid:acyl-CoA transferase protein may be any fatty acid:acyl-CoA transferase protein suitable for the preparation of polyester materials according to the inventive processes. Fatty acid:acyl-CoA transferase proteins include, but are not limited to, 4-hydroxybutyrate:acetyl-CoA transferase from *Clostridium aminobutyricum*, propionate:acyl-CoA transferase from *Clostridium propionicum*, and succinate:acyl-CoA transferase from *Clostridium kluyveri*. Preferably, the fatty acid:acyl-CoA transferase protein is a 4-hydroxybutyrate:acyl-CoA transferase protein, more preferably a *Clostridium kluyveri* 4-hydroxybutyrate:acyl-CoA transferase protein, and most preferably, the *Clostridium kluyveri* 4-hydroxybutyrate:acyl-CoA transferase protein encoded by the *Clostridium kluyveri orfZ* 4-hydroxybutyrate:acyl-CoA transferase structural gene. The nucleic acid segment may comprise deoxyribonucleic acids (i.e. DNA) or ribonucleic acids (i.e. RNA). The nucleic acid segment may further be single or double stranded. The nucleic acid segment may further be linear or circular in conformation. The nucleic acid segment may further comprise a promoter sequence functional in bacterial cells. The promoter may be inducible or constitutive. Promoters functional in bacterial cells are generally known to those of skill in the art, and include, but are not limited to the lac promoter, the bla promoter, the P<sub>L</sub> promoter, the P<sub>trc</sub> promoter, and the T7 promoter.

In an alternative embodiment, the invention provides a nucleic acid segment that encodes a polyhydroxyalkanoic acid synthase protein, and that encodes an acyl-CoA synthetase protein. The polyhydroxyalkanoic acid synthase protein may generally be any polyhydroxyalkanoic acid synthase protein suitable for the production of polyester materials according to the inventive processes. The polyhydroxyalkanoic acid synthase protein may be monomeric or dimeric. Monomeric polyhydroxyalkanoic acid synthase proteins include, but are not limited to, those from *Rhizobium meliloti*, *Alcaligenes eutrophus*, *Alcaligenes sp.*, *Rhizobium etli*, *Paracoccus denitrificans*, *Acinetobacter sp.*, *Rhodobacter sphaeroides*, *Methylobacterium extorquens*, *Pseudomonas oleovorans*, *Pseudomonas aeruginosa*, *Rhodococcus ruber*, and *Zoogloea ramigera*. Dimeric polyhydroxyalkanoic acid synthase proteins include, but are not limited to, those from *Thiocystis violacea* and *Chromatium vinosum*. Preferably, the polyhydroxyalkanoic acid synthase protein is an *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein, and more preferably, the *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein encoded by the *Alcaligenes eutrophus phaC* polyhydroxyalkanoic acid synthase structural gene. The acyl-CoA synthetase protein may be any acyl-CoA synthetase protein suitable for the preparation of polyester materials according to the inventive processes. Sources of acyl-CoA synthetase proteins include, but are not limited to, *Alcaligenes eutrophus*, *Methanothrix soehngenii*, and *Aspergillus nidulans*. Preferably, the acyl-CoA synthetase protein is a thiokinase protein, and more preferably, a 4-hydroxybutyrate thiokinase protein. The nucleic acid segment may comprise deoxyribonucleic acids (i.e. DNA) or ribonucleic acids (i.e. RNA). The nucleic acid segment may further be single or double stranded. The nucleic acid segment may further be linear or circular in conformation. The nucleic acid segment may further comprise a promoter sequence functional in bacterial cells. The promoter may be inducible or constitutive. Promoters functional in bacterial cells are generally known to those of skill in the art, and include, but are not limited to the lac promoter, the bla promoter, the P<sub>L</sub> promoter, the P<sub>trc</sub> promoter, and the T7 promoter.

The invention further provides recombinant vectors comprising a nucleic acid segment, wherein the segment encodes a polyhydroxyalkanoic acid synthase protein and

encodes a fatty acid:acyl-CoA transferase protein. This vector may generally be any vector suitable for the delivery of the nucleic acid into a cell. Vectors are well known to those of skill in the art, and include, but are not limited to, plasmids, artificial chromosomes, viruses, bacteriophage, cosmids, and phagemids. In a preferred embodiment, the vector may be

5 pKSSE5.3 or pSKSE5.3 as disclosed herein.

In a preferred embodiment, the invention encompasses a cell comprising a nucleic acid segment encoding a polyhydroxyalkanoic acid synthase protein and encoding a fatty acid:acyl-CoA transferase protein. The polyhydroxyalkanoic acid synthase protein may generally be any polyhydroxyalkanoic acid synthase protein suitable for the production of

10 polyester materials according to the inventive processes. The polyhydroxyalkanoic acid synthase protein may be monomeric or dimeric. Monomeric polyhydroxyalkanoic acid synthase proteins include, but are not limited to, those from *Rhizobium meliloti*, *Alcaligenes eutrophus*, *Alcaligenes sp.*, *Rhizobium etli*, *Paracoccus denitrificans*, *Acinetobacter sp.*, *Rhodobacter sphaeroides*, *Methylobacterium extorquens*, *Pseudomonas oleovorans*,

15 *Pseudomonas aeruginosa*, *Rhodococcus ruber*, and *Zoogloea ramigera*. Dimeric polyhydroxyalkanoic acid synthase proteins include, but are not limited to, those from *Thiocystis violacea* and *Chromatium vinosum*. Preferably, the polyhydroxyalkanoic acid synthase protein is an *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein, and more preferably, the *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein

20 encoded by the *Alcaligenes eutrophus phaC* polyhydroxyalkanoic acid synthase structural gene. The fatty acid:acyl-CoA transferase protein may be any fatty acid:acyl-CoA transferase protein suitable for the preparation of polyester materials according to the inventive processes. Fatty acid:acyl-CoA transferase proteins include, but are not limited to, 4-

25 hydroxybutyrate:acyl-CoA transferase from *Clostridium aminobutyricum*, propionate:acyl-CoA transferase from *Clostridium propionicum*, and succinate:acyl-CoA transferase from *Clostridium kluyveri*. Preferably, the fatty acid:acyl-CoA transferase protein is a 4-

30 hydroxybutyrate:acyl-CoA transferase protein, more preferably a *Clostridium kluyveri* 4-hydroxybutyrate:acyl-CoA transferase protein, and most preferably, the *Clostridium kluyveri* 4-hydroxybutyrate:acyl-CoA transferase protein encoded by the *Clostridium kluyveri orfZ* 4-

hydroxybutyrate:acyl-CoA transferase structural gene. The cell may generally be any cell

suitable for the preparation of polyester materials. The cell may be, but is not limited to, a plant cell, a mammalian cells, an insect cell, a fungal cell, and a bacterial cell. Preferably, the cell is a plant cell. Preferably, the bacterial cell is *Escherichia coli*, and more preferably, the bacterial cell is *Escherichia coli* strain XL1-Blue.

5           In an alternative embodiment, the invention encompasses a cell comprising a nucleic acid segment encoding a polyhydroxyalkanoic acid synthase protein and encoding an acyl-CoA synthetase protein. The polyhydroxyalkanoic acid synthase protein may generally be any polyhydroxyalkanoic acid synthase protein suitable for the production of polyester materials according to the inventive processes. The polyhydroxyalkanoic acid synthase  
10   protein may be monomeric or dimeric. Monomeric polyhydroxyalkanoic acid synthase proteins include, but are not limited to, those from *Rhizobium meliloti*, *Alcaligenes eutrophus*, *Alcaligenes sp.*, *Rhizobium etli*, *Paracoccus denitrificans*, *Acinetobacter sp.*, *Rhodobacter sphaeroides*, *Methylobacterium extorquens*, *Pseudomonas oleovorans*, *Pseudomonas aeruginosa*, *Rhodococcus ruber*, and *Zoogloea ramigera*. Dimeric polyhydroxyalkanoic acid  
15   synthase proteins include, but are not limited to, those from *Thiocystis violacea* and *Chromatium vinosum*. Preferably, the polyhydroxyalkanoic acid synthase protein is an *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein, and more preferably, the *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein encoded by the *Alcaligenes eutrophus phaC* polyhydroxyalkanoic acid synthase structural gene. The acyl-CoA synthetase  
20   protein may be any acyl-CoA synthetase protein suitable for the preparation of polyester materials according to the inventive processes. Sources of acyl-CoA synthetase proteins include, but are not limited to, *Alcaligenes eutrophus*, *Methanothrix soehngenii*, and *Aspergillus nidulans*. Preferably, the acyl-CoA synthetase protein is a thiokinase protein, and more preferably a 4-hydroxybutyrate thiokinase protein. The cell may generally be any cell  
25   suitable for the preparation of polyester materials. The cell may be, but is not limited to, a plant cell, a mammalian cells, an insect cell, a fungal cell, and a bacterial cell. Preferably, the cell is a plant cell. Preferably, the bacterial cell is *Escherichia coli*, and more preferably, the bacterial cell is *Escherichia coli* strain XL1-Blue.

The invention further discloses methods for the preparation of a transformed cell, the transformed cell being suitable for the preparation of polyester materials. The type of cell includes, but is not limited to, a plant cell, a mammalian cell, an insect cell, a fungal cell, and a bacterial cell. Preferably, the cell is a plant cell. Alternatively, the cell is preferably a  
5 bacterial cell, more preferably the bacterial cell is *Escherichia coli*, and most preferably, the bacterial cell is *Escherichia coli* strain XL1-Blue. Means for the transformation of plants are well known in the art. Methods include, but are not limited to, liposome mediated transformation, electroporation, treatment with chemicals that increase free DNA uptake, free DNA delivery via microparticle bombardment, and transformation using viruses or pollen.

10 Means for the transformation of bacterial cells are also well known in the art. Methods include, but are not limited to, electroporation, calcium chloride mediated transformation, and polyethylene glycol mediated transformation. The disclosed transformation methods comprise selecting a host cell, contacting the host cell with a nucleic acid segment encoding a polyhydroxyalkanoic acid synthase protein and encoding a fatty acid:acyl-CoA transferase  
15 protein, the contacting step being performed under conditions suitable for uptake of the nucleic acid segment by the cell. Subsequent regeneration of the cell affords the transformed cell. The polyhydroxyalkanoic acid synthase protein may generally be any polyhydroxyalkanoic acid synthase protein suitable for the production of polyester materials according to the inventive processes. The polyhydroxyalkanoic acid synthase protein may be  
20 monomeric or dimeric. Monomeric polyhydroxyalkanoic acid synthase proteins include, but are not limited to, those from *Rhizobium meliloti*, *Alcaligenes eutrophus*, *Alcaligenes sp.*, *Rhizobium etli*, *Paracoccus denitrificans*, *Acinetobacter sp.*, *Rhodobacter sphaeroides*, *Methylobacterium extorquens*, *Pseudomonas oleovorans*, *Pseudomonas aeruginosa*, *Rhodococcus ruber*, and *Zoogloea ramigera*. Dimeric polyhydroxyalkanoic acid synthase  
25 proteins include, but are not limited to, those from *Thiocystis violacea* and *Chromatium vinosum*. Preferably, the polyhydroxyalkanoic acid synthase protein is an *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein, and more preferably, the *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein encoded by the *Alcaligenes eutrophus phaC* polyhydroxyalkanoic acid synthase structural gene. The fatty acid:acyl-CoA transferase  
30 protein may be any fatty acid:acyl-CoA transferase protein suitable for the preparation of polyester materials according to the inventive processes. Fatty acid:acyl-CoA transferase

proteins include, but are not limited to, 4-hydroxybutyrate:acetyl-CoA acyltransferase from *Clostridium aminobutyricum*, propionate:acetyl-CoA acyltransferase from *Clostridium propionicum*, and succinate:acetyl-CoA transferase from *Clostridium kluyveri*. Preferably, the fatty acid:acyl-CoA transferase protein is a 4-hydroxybutyrate:acyl-CoA transferase protein, more preferably a *Clostridium kluyveri* 4-hydroxybutyrate:acyl-CoA transferase protein, and most preferably, the *Clostridium kluyveri* 4-hydroxybutyrate:acyl-CoA transferase protein encoded by the *Clostridium kluyveri orfZ* 4-hydroxybutyrate:acyl-CoA transferase structural gene.

The invention discloses alternative methods for the preparation of a transformed cell, the transformed cell being suitable for the preparation of polyester materials. The disclosed transformation methods comprise selecting a host cell, contacting the host cell with a nucleic acid segment encoding a polyhydroxyalkanoic acid synthase protein and encoding an acyl-CoA synthetase protein, the contacting step being performed under conditions suitable for uptake of the nucleic acid segment by the cell. Subsequent regeneration of the cell affords the transformed cell. The type of cell includes, but is not limited to, a plant cell, a mammalian cell, an insect cell, a fungal cell, and a bacterial cell. Preferably, the cell is a plant cell. Alternatively, the cell is preferably a bacterial cell, more preferably the bacterial cell is *Escherichia coli*, and most preferably, the bacterial cell is *Escherichia coli* strain XL1-Blue. Means for the transformation of plants are well known in the art. Methods include, but are not limited to, liposome mediated transformation, electroporation, treatment with chemicals that increase free DNA uptake, free DNA delivery via microparticle bombardment, and transformation using viruses or pollen. Means for the transformation of bacterial cells are also well known in the art. Methods include, but are not limited to, electroporation, calcium chloride mediated transformation, and polyethylene glycol mediated transformation. The polyhydroxyalkanoic acid synthase protein may generally be any polyhydroxyalkanoic acid synthase protein suitable for the production of polyester materials according to the inventive processes. The polyhydroxyalkanoic acid synthase protein may be monomeric or dimeric. Monomeric polyhydroxyalkanoic acid synthase proteins include, but are not limited to, those from *Rhizobium meliloti*, *Alcaligenes eutrophus*, *Alcaligenes sp.*, *Rhizobium etli*, *Paracoccus denitrificans*, *Acinetobacter sp.*, *Rhodobacter sphaeroides*, *Methylobacterium extorquens*,



*Pseudomonas oleovorans*, *Pseudomonas aeruginosa*, *Rhodococcus ruber*, and *Zoogloea ramigera*. Dimeric polyhydroxyalkanoic acid synthase proteins include, but are not limited to, those from *Thiocystis violacea* and *Chromatium vinosum*. Preferably, the polyhydroxyalkanoic acid synthase protein is an *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein, and more preferably, the *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein encoded by the *Alcaligenes eutrophus phaC* polyhydroxyalkanoic acid synthase structural gene. The acyl-CoA synthetase protein may be any acyl-CoA synthetase protein suitable for the preparation of polyester materials according to the inventive processes. Sources of acyl-CoA synthetase proteins include, but are not limited to, *Alcaligenes eutrophus*, *Methanothrix soehngenii*, and *Aspergillus nidulans*. Preferably, the acyl-CoA synthetase protein is a thiokinase protein, and more preferably, a 4-hydroxybutyrate thiokinase protein.

The invention discloses methods for the preparation of polyester materials. The polyester materials may be any polyester material obtained via the inventive processes. The polyester may be a homopolyester or a copolyester. Preferably, the homopolyester is poly (4-hydroxybutyric acid) or poly (3-hydroxybutyric acid). The copolyester is preferably poly (3-hydroxybutyric acid-co- 4-hydroxybutyric acid). In a preferred embodiment, the method comprises the steps of a) obtaining a cell containing a nucleic acid segment, the nucleic acid segment encoding a polyhydroxyalkanoic acid synthase protein and encoding a fatty acid:acyl-CoA transferase protein; b) establishing a culture of the cell; c) culturing the cell under conditions suitable for the production of a polyester; and d) isolating the polyester from the cell. The polyhydroxyalkanoic acid synthase protein may generally be any polyhydroxyalkanoic acid synthase protein suitable for the production of polyester materials according to the inventive method. The polyhydroxyalkanoic acid synthase protein may be monomeric or dimeric. Monomeric polyhydroxyalkanoic acid synthase proteins include, but are not limited to, those from *Rhizobium meliloti*, *Alcaligenes eutrophus*, *Alcaligenes sp.*, *Rhizobium etli*, *Paracoccus denitrificans*, *Acinetobacter sp.*, *Rhodobacter sphaeroides*, *Methylobacterium extorquens*, *Pseudomonas oleovorans*, *Pseudomonas aeruginosa*, *Rhodococcus ruber*, and *Zoogloea ramigera*. Dimeric polyhydroxyalkanoic acid synthase proteins include, but are not limited to, those from *Thiocystis violacea* and *Chromatium*

*vinosum*. Preferably, the polyhydroxyalkanoic acid synthase protein is an *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein, and more preferably, the *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein encoded by the *Alcaligenes eutrophus phaC* polyhydroxyalkanoic acid synthase structural gene. The fatty acid:acyl-CoA transferase protein may be any fatty acid:acyl-CoA transferase protein suitable for the preparation of polyester materials according to the inventive method. Sources of acyl-CoA synthetase proteins include, but are not limited to, *Alcaligenes eutrophus*, *Methanothrix soehngenii*, and *Aspergillus nidulans*. Preferably, the fatty acid:acyl-CoA transferase protein is a 4-hydroxybutyrate:acyl-CoA transferase protein, more preferably a *Clostridium kluyveri* 4-hydroxybutyrate:acyl-CoA transferase protein, and most preferably, the *Clostridium kluyveri* 4-hydroxybutyrate:acyl-CoA transferase protein encoded by the *Clostridium kluyveri orfZ* 4-hydroxybutyrate:acyl-CoA transferase structural gene. The culture may contain glucose or any material capable of conversion to glucose by the cell, as a carbon source. The culture may contain 4-hydroxybutyric acid, the sodium salt of 4-hydroxybutyric acid,  $\gamma$ -butyrolactone, 1,4-butanediol, 4-hydroxyvaleric acid,  $\gamma$ -valerolactone, 1,4-pentanediol, 3-hydroxybutyric acid, the sodium salt of 3-hydroxybutyric acid, a hydroxypropionic acid, a hydroxybutyric acid, a hydroxyvaleric acid, a hydroxycaproic acid, a hydroxyheptanoic acid, a hydroxyoctanoic acid, a hydroxydecanoic acid,  $\gamma$ -caprolactone,  $\gamma$ -heptanolactone,  $\gamma$ -octanolactone,  $\gamma$ -decanolactone, or any material capable of conversion to 4-hydroxybutyric acid by the cell. The culture may contain molecular oxygen. Molecular oxygen may be present due to bubbling of oxygen gas, or an oxygen containing gas, such as air, through the culture. Alternatively, the molecular oxygen may be present due to agitation of the culture. The cell may contain an protein capable of hydrolyzing lactones to the corresponding hydroxyalkanoic acids. Such proteins may include, but are not limited to, the 1,4-lactonase proteins from rat and humans. To facilitate conversion of 2-oxoglutarate to polyester materials, the cell may further comprise a 2-oxoglutarate decarboxylase protein (for example, from *Leuconostoc oenos* or *Euglena gracilis*) and a 4-hydroxybutyrate dehydrogenase protein (for example, from *C. Kluyveri* or *A. eutrophus*). To facilitate conversion of succinate to polyester materials, the cell may further comprise a succinate:acetyl-CoA transferase protein (for example, from *C. kluyveri*), a succinate-semialdehyde dehydrogenase protein (for example, from *C. kluyveri*), and a 4-hydroxybutyrate dehydrogenase protein. To facilitate conversion of succinyl-CoA to

polyester materials, the cell may further comprise a succinate-semialdehyde dehydrogenase protein, and a 4-hydroxybutyrate dehydrogenase protein. To facilitate conversion of propionyl-CoA to polyester materials, the cell may further comprise a 2-methylcitrate synthase protein (for example, from *Saccharomyces cerevisiae*), a 2-methylcitrate dehydratase protein (for example, from *Saccharomyces cerevisiae*), a 2-methylisocitrate dehydratase protein (for example, from *Saccharomyces cerevisiae*), a 2-methylisocitrate lyase protein (for example, from *Saccharomyces cerevisiae*), a succinate:acetyl-CoA transferase protein (for example, from *C. kluyveri*), a succinate-semialdehyde dehydrogenase protein, and a 4-hydroxybutyrate dehydrogenase protein. It will be apparent to those of skill in the art that different combinations of polyhydroxyalkanoic acid synthases, fatty acid:acyl-CoA transferases, and chemical substrates can be used according to the inventive methods to afford polyester materials.

The invention further describes alternative methods for the preparation of polyester materials. In a preferred embodiment, the method comprises the steps of a) obtaining a cell containing a nucleic acid segment, the nucleic acid segment encoding a polyhydroxyalkanoic acid synthase protein and encoding an acyl-CoA synthetase protein; b) establishing a culture of the cell; c) culturing the cell under conditions suitable for the production of a polyester; and d) isolating the polyester from the cell. The polyester materials may be any polyester material obtained via the inventive processes. The polyester may be a homopolyester or a copolyester. Preferably, the homopolyester is poly (4-hydroxybutyric acid) or poly (3-hydroxybutyric acid). The copolyester is preferably poly (3-hydroxybutyric acid -co- 4-hydroxybutyric acid). The polyhydroxyalkanoic acid synthase protein may generally be any polyhydroxyalkanoic acid synthase protein suitable for the production of polyester materials according to the inventive method. The polyhydroxyalkanoic acid synthase protein may be monomeric or dimeric. Monomeric polyhydroxyalkanoic acid synthase proteins include, but are not limited to, those from *Rhizobium meliloti*, *Alcaligenes eutrophus*, *Alcaligenes sp.*, *Rhizobium etli*, *Paracoccus denitrificans*, *Acinetobacter sp.*, *Rhodobacter sphaeroides*, *Methylobacterium extorquens*, *Pseudomonas oleovorans*, *Pseudomonas aeruginosa*, *Rhodococcus ruber*, and *Zoogloea ramigera*. Dimeric polyhydroxyalkanoic acid synthase proteins include, but are not limited to, those from *Thiocystis violacea* and *Chromatium vinosum*. Preferably, the

polyhydroxyalkanoic acid synthase protein is an *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein, and more preferably, the *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein encoded by the *Alcaligenes eutrophus phaC* polyhydroxyalkanoic acid synthase structural gene. The acyl-CoA synthetase protein may be any acyl-CoA synthetase protein suitable for the preparation of polyester materials according to the inventive processes. Sources of acyl-CoA synthetase proteins include, but are not limited to, *Alcaligenes eutrophus*, *Methanothrix soehngenii*, and *Aspergillus nidulans*. Preferably, the acyl-CoA synthetase protein is a thiokinase protein, and more preferably, a 4-hydroxybutyrate thiokinase protein. The culture may contain glucose or any material capable of conversion to glucose by the cell, as a carbon source. The culture may contain 4-hydroxybutyric acid, the sodium salt of 4-hydroxybutyric acid,  $\gamma$ -butyrolactone, 1,4-butanediol, 4-hydroxyvaleric acid,  $\gamma$ -valerolactone, 1,4-pentanediol, 3-hydroxybutyric acid, the sodium salt of 3-hydroxybutyric acid, a hydroxypropionic acid, a hydroxybutyric acid, a hydroxyvaleric acid, a hydroxycaproic acid, a hydroxyheptanoic acid, a hydroxyoctanoic acid, a hydroxydecanoic acid,  $\gamma$ -caprolactone,  $\gamma$ -heptanolactone,  $\gamma$ -octanolactone,  $\gamma$ -decanolactone, or any material capable of conversion to 4-hydroxybutyric acid by the cell. The culture may contain molecular oxygen. Molecular oxygen may be present due to bubbling of oxygen gas, or an oxygen containing gas, such as air, through the culture. Alternatively, the molecular oxygen may be present due to agitation of the culture. The cell may contain an enzyme capable of hydrolyzing lactones to the corresponding hydroxyalkanoic acids. Such proteins may include, but are not limited to, the 1,4-lactonase proteins from rat and humans. To facilitate conversion of 2-oxoglutarate to polyester materials, the cell may further comprise a 2-oxoglutarate decarboxylase protein and a 4-hydroxybutyrate dehydrogenase protein. To facilitate conversion of succinate to polyester materials, the cell may further comprise a succinate:acetyl-CoA transferase protein, a succinate-semialdehyde dehydrogenase protein, and a 4-hydroxybutyrate dehydrogenase protein. To facilitate conversion of succinyl-CoA to polyester materials, the cell may further comprise a succinate-semialdehyde dehydrogenase protein, and a 4-hydroxybutyrate dehydrogenase protein. To facilitate conversion of propionyl-CoA to polyester materials, the cell may further comprise a 2-methylcitrate synthase protein, a 2-methylcitrate dehydratase protein, a 2-methylisocitrate dehydratase protein, a 2-methylisocitrate lyase protein, a succinate:acetyl-CoA transferase protein, a succinate-semialdehyde dehydrogenase protein,

and a 4-hydroxybutyrate dehydrogenase protein. It will be apparent to those of skill in the art that different combinations of polyhydroxyalkanoic acid synthases, acyl-CoA synthetases, and chemical substrates can be used according to the inventive methods to afford polyester materials.

5 In a further alternative embodiment, the preparation of polyester materials may be achieved by the co-expression of a polyhydroxyalkanoic acid synthase protein, an acyl kinase protein, and a phosphotransacylase protein. A source of acyl kinase includes, but is not limited to, the butyrate kinase from *Clostridium acetobutylicum*. A source of a phosphotransacylase protein includes, but is not limited to, the phosphotransbutyrylase protein  
10 from *Clostridium acetobutylicum*.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for  
15 its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

## EXAMPLES

### 20 **Materials and methods**

#### Microbiological Deposit

A deposit according to the Budapest Treaty of *Escherichia coli* XL1-Blue containing the recombinant plasmid pKSSE5.3 (accession number DSM 11427) and of *Escherichia coli* XL1-Blue containing the recombinant plasmid pSKSE5.3 (accession number DSM 11435)  
25 was made on February 24, 1997 with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Mascheroder Weg 1b, D-38124 Braunschweig, Germany.

### Bacterial strains and plasmids

*Escherichia coli* strain XL1-Blue (Bullock, W.O. et al. (1987) *BioTechniques* 5:376-378) and the plasmids pBluescriptKS<sup>+</sup> and pBluescriptSK<sup>+</sup> (Stratagene, La Jolla, CA), pSK2665 (Schubert, P. et al. (1991) *J. Bacteriol.* 173:168-175) and pCK3pSK (Söhling, B. and Gottschalk, G. (1996) *J. Bacteriol.* 178:871-880) were used.

### Media and cultivation conditions

Cells of *Escherichia coli* were cultivated at 37°C in complex Luria-Bertani broth or in M9-minerals salts medium supplemented with 0.02% (w/vol) yeast extract (Sambrook, J. et al. (1989) *Molecular cloning; a laboratory manual*. 2nd edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). The addition of antibiotics prepared according to Sambrook, J. et al. (1989), and of carbon sources, which were sterilized by filtration, are described in the text. Cultivations were performed either on solidified media, which were obtained by the addition of 1.5% (wt/vol) agar, or in liquid media in erlenmeyer flasks incubated on a rotary shaker.

### Isolation and analysis of polyesters

For quantitative determination of polyhydroxyalkanoic acid and for the analysis of the constituents of polyhydroxyalkanoic acid, 3 - 5 mg of lyophilized cell material or the isolated polyester were subjected to methanolysis in the presence of 15% (vol/vol) sulfuric acid. The resulting hydroxyacyl methylesters were analyzed by gas chromatography as described in detail by Brandl, H. et al. (1988, *Appl. Environ. Microbiol.* 54:1977-1982) and Timm, A. et al. (1990, *Appl. Microbiol. Biotechnol.* 33:296-301).

Polyhydroxyalkanoic acids were isolated from lyophilized cells by extraction with chloroform in a soxhlet apparatus. The polyester was precipitated from the chloroform solution by the addition of 10 volumes of ethanol, and the precipitate was subsequently separated from the solvents by filtration. Remaining solvents were removed by exposure of the polyester to a stream of air. For further purification, the polyester was dissolved in chloroform and the precipitation with ethanol was repeated.

### Nucleic acid techniques

Plasmid DNA and DNA restriction fragments were isolated and analyzed by standard methods (Sambrook, J. et al. (1989) Molecular cloning; a laboratory manual. 2nd edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). Restriction enzymes, ligases, and other enzymes were used according to the manufacturer's instructions.

### Physical characterization of poly(4-hydroxybutyric acid) samples

Analysis of polymer composition was performed using gas chromatography methods as described by Braunegg, G. et al. (1978, *Eur. J. Appl. Microbiol.* 6:29-37). Analysis of molecular weight and polydispersity was performed by gel permeation chromatography (GPC, Ishihara, Y. et al. (1996) *J. Ferm. Bioeng.* 81:422-428). Analysis of melting points, rate of crystallization,  $dH_m$ , and  $E_a$  were performed by differential scanning calorimetry (DSC, Kemnitzer, J.E. et al. (1995) *J. Env. Polym. Degrad.* 3:37-47).

### **EXAMPLE 1: Construction of plasmids**

A 3.5-kbp *SmaI/ApaI* restriction fragment comprising the entire polyhydroxyalkanoic acid synthase structural gene (*phaC<sub>Ae</sub>*, GenBank Accession number J05003, Peoples, O.P. and Sinskey, A.J. (1989) *J. Biol. Chem.* 264:15298-15303) plus 878 of 1,221 bp of the 5' region of the  $\beta$ -ketothiolase structural gene from *A. eutrophus*, referred to as SA35, was isolated from the hybrid plasmid pSK2665 that had been cloned previously (Schubert, P. et al. (1991) *J. Bacteriol.* 173:168-175). In addition, a 1.8 kb *ApaI/EcoRI* restriction fragment comprising the entire *orfZ<sub>Ck</sub>* (*phaA'<sub>Ae</sub>*, GenBank Accession number L21902, Söhling, B. and Gottschalk, G. (1993) *J. Bacteriol.* 178:871-880) from *C. kluverii*, and referred to as AE18, was isolated from the hybrid plasmid pCK3pSK that had been cloned previously (Söhling, B. and Gottschalk, G. (1996) *J. Bacteriol.* 178:871-880). Both fragments were ligated to *EcoRI/SmaI* digested pBluescript vectors KS<sup>-</sup> and SK<sup>-</sup>. The ligation products (pKSSE5.3 and pSKSE5.3, respectively) were transformed into *Escherichia coli* strain XL1-Blue using calcium chloride methodologies (Sambrook, J. et al. (1989) Molecular cloning; a laboratory

manual. 2nd edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). Plasmid pKSSE5.3 contains *phaC<sub>Ae</sub>* and *orfZ<sub>Ck</sub>* adjacent but antilinear to the *lacZ* promoter. In pSKSE5.3, *phaC<sub>Ae</sub>* and *orfZ<sub>Ck</sub>* were located downstream and colinear to the *lacZ* promoter. All constructs were analyzed by agarose gel electrophoresis for the presence of the expected restriction fragments.

For control experiments, fragment SA35 was ligated to pBluescript KS<sup>+</sup> or to pBluescript SK<sup>+</sup> resulting in the plasmids pKSSA35 (*phaC<sub>Ae</sub>* plus *phaA'<sub>Ae</sub>* adjacent but antilinear to the *lacZ* promoter) or pSKSA35 (*phaC<sub>Ae</sub>* plus *phaA'<sub>Ae</sub>* downstream and colinear to the *lacZ* promoter), respectively. Similarly, fragment AE18 alone was ligated to pBluescript KS<sup>+</sup> or to pBluescript SK<sup>+</sup> resulting in the plasmids pKSAE18 (*orfZ<sub>Ck</sub>* downstream and colinear to the *lacZ* promoter) and pSKAE18 (*orfZ<sub>Ck</sub>* adjacent but antilinear to the *lacZ* promoter), respectively.

**EXAMPLE 2:** Synthesis of poly(4-hydroxybutyric acid) from 4-hydroxybutyric acid in recombinant strains of *Escherichia coli*

The recombinant strains of *Escherichia coli* XL1-Blue were cultivated at 37°C as batch cultures in erlenmeyer flasks either using LB complex medium supplemented with 4-hydroxybutyric acid alone or in combination with glucose as an additional carbon source, or M9 mineral salts medium containing 4-hydroxybutyric acid and glucose as carbon sources. The hybrid plasmids pKSSE5.3 and pSKSE5.3, which comprised *phaC<sub>Ae</sub>*, *phaA'<sub>Ae</sub>* plus *orfZ<sub>Ck</sub>*, conferred to *Escherichia coli* the capability to synthesize and accumulate a homopolyester of 4-hydroxybutyric acid if the strains were cultivated in the presence of 4-hydroxybutyric acid plus glucose as carbon source (Table 1).



**Table 1.** Accumulation of poly(4-hydroxybutyric acid) by recombinant strains of *Escherichia coli* XL1-Blue from 4-hydroxybutyric acid plus glucose

<u>Medium</u>	<u>Carbon source</u>	<u>volume of medium (mL)</u>	<u>PHA content (% of CDW)</u>	<u>accumulated polyester</u>
<u>with pSKSE5.3</u>				
LB + IPTG	0.5% glucose	50	3.3	poly (4HB)
	0.4% 4HB (sodium salt)			
LB + IPTG	0.5% glucose	100	10.5	poly (4HB)
	0.4% 4HB (sodium salt)			
LB + IPTG	0.5% glucose	150	8.8	poly (4HB)
	0.4% 4HB (sodium salt)			
M9 + IPTG	0.5% glucose	100	16.9	poly (4HB)
	0.4% 4HB (sodium salt)			
<u>with pKSSE5.3</u>				
LB	0.5% glucose	50	4.3	poly (4HB)
	0.4% 4HB (sodium salt)			
LB	0.5% glucose	100	40.8	poly (4HB)
	0.4% 4HB (sodium salt)			
LB	0.5% glucose	150	20.4	poly (4HB)
	0.4% 4HB (sodium salt)			
M9	0.5% glucose	100	58.5	poly (4HB)
	0.4% 4HB (sodium salt)			
<u>with pSKSA35</u>				
LB + IPTG	0.5% glucose	50-150	nd	-
	0.4% 4HB (sodium salt)			
M9 + IPTG	0.5% glucose	100	nd	-
	0.4% 4HB (sodium salt)			
<u>with pKSAE18</u>				
LB + IPTG	0.5% glucose	50-150	nd	-
	0.4% 4HB (sodium salt)			
M9 + IPTG	0.5% glucose	100	nd	-
	0.4% 4HB (sodium salt)			

Cells were cultivated at 37°C for 72 hours in one-stage cultivation experiments in 250 mL erlenmeyer flasks as described in the text that were incubated on a rotary shaker (220 rpm).

- 5 The cultures were inoculated with 0.04 vol of an overnight preculture in Luria Bertani broth containing 0.5% (wt/vol) glucose plus 0.4% (wt/vol) 4-hydroxybutyric acid. M9 medium was supplemented with 0.02% (wt/vol) yeast extract. At the end of the experiment, the cells were harvested, washed with tap water, lyophilized and analyzed for PHA content and composition by gas chromatography.

10

Abbreviations and symbols: IPTG, isopropyl-1-thio-β-D-galactopyranoside; nd, not detectable; -, not relevant.

Light microscopic examination of the cells revealed cytoplasmic inclusions, and the harvested cells were more opaque in appearance than were strains not harboring pKSSE5.3 or pSKSE5.3. If glucose was omitted as a cosubstrate, the amount of polyhydroxyalkanoic acid in the cells was approximately in the same range but consisting of a copolyester of 3-hydroxybutyric acid and 4-hydroxybutyric acid, poly(3-hydroxybutyric acid-*co*-4-hydroxybutyric acid), rather than poly(4-hydroxybutyric acid). Early in the growth, only 4-hydroxybutyric acid was incorporated into the polyhydroxyalkanoic acid. However, after approximately one day of cultivation, the cells began to incorporate increasing amounts of 3-hydroxybutyric acid, and after four days, the molar fraction of 3-hydroxybutyric acid had increased to approximately 70% (Table 2). If glucose was provided as the sole carbon source, no polyhydroxyalkanoic acid was accumulated. The addition of 4-hydroxybutyric acid to the medium as a carbon source appeared essential to obtain poly(4-hydroxybutyric acid), and glucose provided as a cosubstrate appeared to limit the incorporation of 3-hydroxybutyric acid into the polyhydroxyalkanoic acid.

**Table 2.** Accumulation of poly(3-hydroxybutyric acid-co-4-hydroxybutyric acid) by recombinant strains of *Escherichia coli* XL1-Blue from 4-hydroxybutyric acid

<u>Strain and</u> <u>Carbon source(s)</u>	<u>Incubation</u> <u>time (hours)</u>	<u>PHA content</u> <u>(% of CDW)</u>	<u>PHA</u> <u>composition</u> <u>mole % 3HB</u>	<u>PHA</u> <u>composition</u> <u>mole % 4HB</u>
<u><i>E. coli</i> XL1-Blue (pSKSE5.3)</u>				
in LB medium with 0.5% glucose and 0.4% Na-4-hydroxybutyrate	20	13.3	nd	100
	44	13.1	11	89
	72	11.1	55	45
	94	15.1	64	36
in LB medium with 0.4% Na-4-hydroxybutyrate	20	20.2	nd	100
	44	20.6	72	28
	72	10.2	61	39
	94	21.9	66	34
<u><i>E. coli</i> XL1-Blue (pKSSE5.3)</u>				
in LB medium with 0.5% glucose and 0.4% Na-4-hydroxybutyrate	20	14.6	nd	100
	44	13.6	nd	100
	72	7.0	nd	100
	94	15.9	7	93
in LB medium with 0.4% Na-4-hydroxybutyrate	20	22.9	nd	100
	44	21.4	62	38
	72	23.7	63	37
	94	29.8	64	36

Cells were cultivated at 37°C in 50 mL Luria Bertani complex medium, which contained the indicated carbon source, in 300 mL Erlenmeyer flasks. At the indicated times, 10 mL samples were withdrawn and subjected to gas chromatographic analysis of the polyester content and composition.

Abbreviations and symbols: nd, not detectable; PHA, polyhydroxyalkanoic acids; 3HB, 3-hydroxybutyric acid; 4HB, 4-hydroxybutyric acid; LB Luria Bertani; CDW, cellular dry weight

Gas chromatographic analysis of the derivatives obtained from washed and lyophilized whole cells gave two major compounds in the chromatograms exhibiting retention times of 20.1 and 9.3 and one minor compound exhibiting a retention time of 24.1 min. These compounds represented most probably  $\gamma$ -butyrolactone, the methylester of 4-hydroxybutyric

acid and the methyl ether of 4-hydroxybutyric acid, respectively, that were also obtained if only 4-hydroxybutyric acid were subjected to acidic methanolysis.

The amount of poly(4-hydroxybutyric acid) accumulated by the cells depended upon the plasmid present in the recombinant *Escherichia coli* XL1-Blue, the medium, and the cultivation conditions. Recombinant strains harboring plasmid pKSSE5.3 accumulated significantly more poly(4-hydroxybutyric acid) as compared to those harboring pSKSE5.3 in LB complex medium as well as in M9 mineral salts medium (Table 1). The addition of IPTG to cultures of *Escherichia coli* XL1-Blue harboring pSKSE5.3 did not significantly influence the amount of polyester produced. With either plasmid, the amount of poly(4-hydroxybutyric acid) accumulated by the cells was always higher in M9-mineral salts medium than in LB complex medium. Supplementation of oxygen to the cultures seems to be crucial for the amount of poly(4-hydroxybutyric acid) accumulated by the cells. This became obvious from experiments in which the ratio of the medium volume to the volume of the erlenmeyer flask was varied. When experiments were performed in a 250 mL erlenmeyer flask, the amount of poly(4-hydroxybutyric acid) increased tremendously when the volume of the medium was increased from 50 to 100 mL independently whether the recombinant strains harbored pSKSE5.3 or pKSSE5.3 (Table 1). If the volume of the medium was further increased to 150 mL, the amount of accumulated poly(4-hydroxybutyric acid) decreased.

All plasmids, which contained only *phaC<sub>Ae</sub>* plus *phaA'<sub>Ae</sub>* (pKSSA35 and pSKSA35) or only *orfZ<sub>Ck</sub>* (pKSAE18 and pSKAE18), did not confer to *Escherichia coli* the capability to synthesize detectable poly(4-hydroxybutyric acid) or of any other polyhydroxyalkanoic acid if 4-hydroxybutyric acid or glucose alone or in combination were used as carbon sources and independent from the volume of the medium or whether IPTG was added or not.

None of the recombinant strains of *Escherichia coli* obtained in this study were able to grow in liquid or on solidified M9 minerals salts medium containing 4-hydroxybutyric acid as the sole carbon source.

**EXAMPLE 3:** Properties of poly(4-hydroxybutyric acid) isolated from recombinant

*Escherichia coli*

In order to confirm accumulation of poly(4-hydroxybutyric acid) by the recombinant strains and to isolate the polyester from the cells, *Escherichia coli* XL1-Blue (pKSSE5.3) was cultivated in M9 mineral salts medium on a larger scale. 2 L baffled erlenmeyer flasks containing 1700 mL medium supplemented with 0.4% (wt/vol) sodium 4-hydroxybutyrate and 1% or 2% glucose were inoculated with a preculture of 50 mL cells and incubated on a rotary shaker for 72 hours. This afforded approximately 1.9 g or 2.5 g, respectively, of dried cells. Gas chromatographic analysis of the whole cells indicated a poly(4-hydroxybutyric acid) content of approximately 80% (wt/wt). From these cells, 0.8 g or 1.1 g of polyhydroxyalkanoic acid could be extracted with chloroform and precipitated with ethanol, respectively. Therefore, only 52% or 55% of the polyester were recovered from the cells. This discrepancy could be explained by incomplete extractions of the cells since the extracted cells still contained poly(4-hydroxybutyric acid) and by losses during the precipitation of the polymer. The isolated material gave in the gas chromatogram only those signals indicative of 4-hydroxybutyric acid. Thus it was confirmed that the cells had accumulated poly(4-hydroxybutyric acid) homopolyester.

Poly(4-hydroxybutyric acid) produced by a polyhydroxyalkanoic acid -leaky mutant of *A. eutrophus* JMP222 and recombinant strains of this mutant harboring extra copies of the *A. eutrophus* PHA synthase operon (Steinbüchel, A. et al. (1994) *J. Environ. Polymer Degrad.* 2:67-74) were observed to be extracted from lyophilized cells at a lower rate than was poly (3-hydroxybutyric acid). Similarly, the poly(4-hydroxybutyric acid) produced by the recombinant strain of *Escherichia coli* employed in this study was extracted from lyophilized cells at a much lower rate than, for example poly(3-hydroxybutyric acid). Poly(4-hydroxybutyric acid) from the recombinant strain precipitated in the presence of an excess of ethanol from the chloroform solution as a highly fibrous material which easily and almost quantitatively ended up on a glass rod if the latter was used for stirring during precipitation. Poly(4-hydroxybutyric acid) from the strains of *A. eutrophus* JMP222 (Steinbüchel, A. et al. (1994) *J. Environ. Polymer Degrad.* 2:67-74) was isolated as a white and elastic material.

Gel permeation chromatography experiments performed with two samples of poly(4-hydroxybutyric acid) homopolyester isolated from two independent batches of cells of recombinant strains of *E. coli* revealed molecular weights ( $M_w$ ) of  $1.75 \times 10^6$  and  $1.85 \times 10^6$ , respectively, with relative low polydispersities ( $M_w/M_n$ ) of 1.45 and 1.48, respectively.

- 5 Therefore, the molecular weights of these poly(4-hydroxybutyric acid) samples were significantly higher than the molecular weights of poly(4-hydroxybutyric acid) isolated from strains of *A. eutrophus* JMP222. In addition, the polydispersity of these samples was much lower (Steinbüchel, A. et al. (1994) *J. Environ. Polymer Degrad.* 2:67-74). The two poly(4-hydroxybutyric acid) samples exhibited melting points ( $T_m$ ) of 67.6 and 63.1°C. The values  
10 for  $dH_m$  and  $E_a$  for the two samples were 45.7 and 44.3 J/g or 69.8 and 83.8 KJ/mol, respectively. At 70°C both polyester samples exhibited a slow rate for crystallization (> 30 min).

**EXAMPLE 4:** Synthesis of polyhydroxyalkanoic acid from  $\gamma$ -butyrolactone, levulinic acid, 4-hydroxyvaleric acid or  $\gamma$ -valerolactone in recombinant strains of *Escherichia coli*

- 15 Carbon sources related to 4-hydroxybutyric acid were also investigated with respect to the formation of polyhydroxyalkanoic acid by the recombinant strains. For this purpose, cells were cultivated in two different stages. In the first stage the cells were grown for 64 hours in the complete M9 minerals salts medium containing 0.5% (wt/vol) glucose plus 0.1% (wt/vol) sodium 4-hydroxybutyrate as carbon sources. These cells were then washed with fresh M9  
20 mineral salts medium and transferred to 250 mL erlenmeyer flasks containing 100 mL ammonium-free M9 mineral salts medium. The density of the cell suspension was diluted approximately 1:1 as compared to the density in the preculture. In the second stage the cells were cultivated for 72 hours on a rotary shaker. If *Escherichia coli* XL1-Blue (pKSSE5.3) was cultivated in M9 mineral salts medium containing 0.5% (wt/vol) glucose plus 0.4%  
25 (wt/vol)  $\gamma$ -butyrolactone as carbon sources, only low amounts of polyhydroxyalkanoic acid (usually below 10% of CDW, wt/wt) were accumulated (Table 3). However, these cells did not accumulate poly(4-hydroxybutyric acid) homopolyester but synthesized a copolyester consisting of 3-hydroxybutyric acid and 4-hydroxybutyric acid, with 4-hydroxybutyric acid as a minor constituent (usually below 30%, mol/mol).

**Table 3.** Cultivation of *E. coli* XL1-Blue (pKSSE5.3) on other precursor substrates for the incorporation of 4-hydroxyalkanoic acids in PHA

<u>Medium</u>	<u>Carbon source</u>	<u>PHA content</u> <u>(% of CDW)</u>	<u>PHA composition (mole %)</u>			
			<u>3HB</u>	<u>3HV</u>	<u>4HB</u>	<u>4HV</u>
<u>two-stage cultivation experiments</u>						
M9*	0.5% glucose	9.6	71	nd	29	nd
	0.4% $\gamma$ -butyrolactone					
M9*	0.5% glucose	4.2	100	nd	nd	nd
	0.4% Na-levulinate					
M9*	0.5% glucose	1.5	100	nd	nd	nd
	0.4% Na-4-hydroxyvalerate					
M9*	0.5% glucose	2.7	100	nd	nd	nd
	0.4% $\gamma$ -valerolactone					
<u>one-stage cultivation experiments</u>						
M9	0.5% glucose	8.0	nd	nd	100	nd
	0.1% $\gamma$ -butyrolactone					
M9	0.5% glucose	13.9	nd	nd	100	nd
	0.2% $\gamma$ -butyrolactone					
M9	0.5% glucose	16.1	nd	nd	100	nd
	0.4% $\gamma$ -butyrolactone					
M9	0.5% glucose	nd	nd	nd	nd	nd
	0.2% Na-levulinate					
M9	0.5% glucose	nd	nd	nd	nd	nd
	0.4% Na-levulinate					

Cells were cultivated at 37°C for 72 hours in one- or two-stage cultivation experiments as described in the text. At the end of the experiment the cells were harvested, washed with tap water, lyophilized and analyzed for PHA content and composition by gas chromatography.

Abbreviations and symbols: M9, regular mineral salts medium supplemented with 0.02% (wt/vol) yeast extract; M9\*, ammonium-free M9 mineral salts medium; nd, not detectable

If in these experiments  $\gamma$ -butyrolactone was replaced by 0.4% (wt/vol) sodium levulinate or by 0.2% (wt/vol) sodium 4-hydroxyvalerate or by 0.2% (wt/vol)  $\gamma$ -valerolactone, only very low amounts of polyhydroxyalkanoic acid never exceeding 4% of the CDW were synthesized and accumulated. Gas chromatographic analysis revealed 3-hydroxybutyric acid

as the only constituent; 4-hydroxybutyric acid, 3-hydroxyvaleric acid (3HV) or 4-hydroxyvaleric acid (4HV) were not detected (Table 3).

Cells of *Escherichia coli* XL1-Blue (pKSSE5.3) were also cultivated in single stage experiments as batch cultures in 250 mL erlenmeyer flasks containing 100 mL complete M9 minerals salts medium plus 0.5% (wt/vol) glucose plus 0.1 to 0.4% (wt/vol)  $\gamma$ -butyrolactone as carbon sources. The cells accumulated significantly more polyhydroxyalkanoic acid, and the polyester consisted of 4-hydroxybutyric acid as the only detectable constituent (Table 3).

With 0.4% (wt/vol) sodium 4-hydroxybutyrate in the medium, for example, poly(4-hydroxybutyric acid) contributed 16.1% (wt/wt) to the CDW. If sodium levulinate instead of sodium 4-hydroxybutyrate was used as a second carbon source in addition to 0.5% (wt/vol) glucose, no polyhydroxyalkanoic acid was detected in the cells.

#### EXAMPLE 5: Production of polyester materials in plants

In plants, transformation vectors capable of introducing bacterial genes involved in polyester biosynthesis can be designed. Generally, such vectors comprise one or more coding sequences of interest under the transcriptional control of 5' and 3' regulatory sequences, including a promoter, and a selectable marker. Typical regulatory sequences include a transcription initiation start site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal. Plant promoter can be inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific. Often-used promoters include the CaMV 35S promoter (Odell, J.T. et al. (1985) *Nature* 313:810-812), the enhanced CaMV 35S promoter, the Figwort Mosaic Virus (FMV) promoter (Richins, R.D. et al. (1987) *NAR* 20:8451-8466), the mannopine synthase (*mas*) promoter, the nopaline synthase (*nos*) promoter, and the octopine synthase (*ocs*) promoter. Useful inducible promoters include heat-shock promoters (Ou-Lee et al. (1986) *Proc. Natl. Acad. Sci. USA* 83:6815; a nitrate-inducible promoter derived from the spinach nitrite reductase gene (Back, E. et al. (1991) *Plant Mol. Biol.* 17:9-18); hormone-inducible promoters (Yamaguchi-, K. et al. (1990) *Plant Mol. Biol.* 15:905-912; Kares, C. et al. (1990) *Plant Mol. Biol.* 15:225-236), and light-inducible promoters associated with the small subunit of RuBP carboxylase and LHCP gene families (Kuhlemeier, C. et al. (1989) *Plant Cell* 1:471-478; Feinbaum, R.L. et al.



(1991) *Mol. Gen. Genet.* 226:449-456; Weisshaar, B. et al. (1991) *EMBO J.* 10:1777-1786; Lam, E. and Chua, N.-H. (1990) *Science* 248:471-474; Castresana, C. et al. (1988) *EMBO J.* 7:1929-1936; Schulze-Lefert, P. et al. (1989) *EMBO J.* 8:651-656). Examples of useful tissue-specific, developmentally-regulated promoters include the  $\beta$ -conglycinin 7S promoter (Doyle, J.J. et al. (1986) *J. Biol. Chem.* 261:9228-9238; Slighton and Beachy (1987) *Planta* 172:356), and seed-specific promoters (Knutzon, D.S. et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:2624-2628; Bustos, M.M. et al. (1991) *EMBO J.* 10:1469-1480; Lam, E. and Chua, N.-H. (1991) *Science* 248:471-474; Stayton, M. et al. (1991) *Aust. J. Plant. Physiol.* 18:507-518). Plant functional promoters useful for preferential expression in seed plastids include those from plant storage protein genes and from genes involved in fatty acid biosynthesis in oilseeds. Examples of such promoters include the 5' regulatory regions from such genes as napin (Kridl et al. (1991) *Seed Sci. Res.* 1:209), phaseolin, zein, soybean trypsin inhibitor, ACP, stearyl-ACP desaturase, and oleosin. Seed-specific gene regulation is discussed in EP 0 255 378. Promoter hybrids can also be constructed to enhance transcriptional activity (Hoffman, U.S. Patent No. 5,106,739), or to combine desired transcriptional activity and tissue specificity. Representative vectors often comprise, operatively linked in sequence in the 5' to 3' direction, a promoter sequence that directs the transcription of a downstream heterologous structural nucleic acid in a plant; optionally, a non-translated leader sequence; a nucleotide sequence that encodes a protein of interest; and a 3' non-translated region that encodes a polyadenylation signal which functions in plant cells to cause the termination of transcription and the addition of polyadenylate nucleotides to the 3' end of the mRNA encoding said protein. Additionally, a factor to consider is the timing and intracellular localization of proteins necessary for the biosynthesis of polyesters. For example, if fatty acid biosynthetic pathways are utilized in oilseed plants such as canola, then polyester biosynthetic protein expression should be concurrent with fatty acid biosynthesis and targeted to the seed leucoplast or leaf chloroplast.

A variety of different methods can be employed to introduce such vectors into plant protoplasts, cells, callus tissue, leaf discs, meristems, etcetera, to generate transgenic plants, including *Agrobacterium*-mediated transformation, particle gun delivery, microinjection, electroporation, polyethylene glycol-mediated protoplast transformation, liposome-mediated

transformation, etcetera (reviewed in Potrykus, I. (1991) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 42:205-226). In general, transgenic plants comprising cells containing and expressing polyhydroxyalkanoic acid synthase and fatty acid:acyl-CoA transferase-encoding nucleic acid can be produced by transforming plant cells with a nucleic acid construct as described above via any of the foregoing methods; selecting plant cells that have been transformed on a selective medium; regenerating plant cells that have been transformed to produce differentiated plants; and selecting a transformed plant which expresses the polyhydroxyalkanoic acid synthase and fatty acid:acyl-CoA transferase -encoding nucleotide sequences.

The encoding nucleic acids can be introduced either in a single transformation event (all necessary DNAs present on the same vector), a co-transformation event (all necessary nucleic acids present on separate vectors that are introduced into plants or plant cells simultaneously), by independent transformation events (all necessary nucleic acids present on separate vectors that are introduced into plants or plant cells independently) or by re-transformation (transforming an already transformed line generated by a single transformation, co-transformation, or independent transformation events). Traditional breeding methods, when applicable, can subsequently be used to incorporate the entire pathway into a single plant. Successful production of the PHA polyhydroxybutyrate in cells of *Arabidopsis* has been demonstrated by Poirier, Y. et al. (1992, *Science* 256:520-523), and in plastids thereof by Nawrath et al. (1994, *Proc. Natl. Acad. Sci. USA* 91:12760-12764).

Specific methods for transforming a wide variety of dicots and obtaining transgenic plants are well documented in the literature (see Gasser, C.S. and Fraley, R.T. (1989) *Science* 244:1293-1299; Fisk, H.J. and Dandekar, A.M. (1993) *Scientia Horticulturae* 55:5-36; Christou (1994) *Agro Food Industry Hi Tech* (March/April 1994) p.17, and the references cited therein).

Successful transformation and plant regeneration have been achieved in the monocots as follows: asparagus (*Asparagus officinalis*; Bytebier et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:5345 ); barley (*Hordeum vulgare*; Wan, Y. and Lemaux, P.G. (1994) *Plant Physiol.*

104:37-48); maize (*Zea mays*; Rhodes, C.A. et al. (1988) *Science* 240:204-207; Gordon-Kamm, W.J. et al. (1990) *Plant Cell* 2:603-618; Fromm, M.E. et al. (1990) *Bio/Technology* 8:833-839; Koziel et al. (1993) *Bio/Technology* 11:194); oats (*Avena sativa*; Somers et al. (1992) *Bio/Technology* 10:1589); orchardgrass (*Dactylis glomerata*; Horn, M.E. et al. (1988) *Plant Cell Rep.* 7:469-472); rice (*Oryza sativa*, including indica and japonica varieties; Toriyama, K. et al. (1988) *Bio/Technology* 6:1072-1074; Zhang, H.M. et al. (1988) *Plant Cell Rep.* 7:379-384; Luo and Wu (1988) *Plant Mol. Biol. Rep.* 6:165; Zhang, W. and Wu, R. (1988) *Theor. Appl. Genet.* 76:835-840; Christou et al. (1991) *Bio/Technology* 9:957); rye (*Secale cereale*; De la Pena et al. (1987) *Nature* 325:274); sorghum (*Sorghum bicolor*; Casas, A.M. et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:11212-11216); sugar cane (*Saccharum* spp.; Bower and Birch (1992) *Plant J.* 2:409); tall fescue (*Festuca arundinacea*; Wang, Z.Y. et al. (1992) *Bio/Technology* 10:691-696); turfgrass (*Agrostis palustris*; Zhong, H. et al. (1993) *Plant Cell Rep.* 13:1-6); and wheat (*Triticum aestivum*; Vasil et al. (1992) *Bio/Technology* 10:667; Weeks, J.T. et al. (1993) *Plant Physiol.* 102:1077-1084; Becker, D. et al. (1994) *Plant J.* 5:299-307).

Particularly useful plants for polyester production include those, such as potato and sugarbeet, that produce carbon substrates which can be employed for polyester biosynthesis. Cereal plants such as corn, wheat, and rice are also preferred. Other useful plants include tobacco and high oil seed plants such as soybean, canola, oil seed rape, *Arabidopsis* sp. and peanut. Plants that grow in desert or in mineralized soil can also be employed for the production of polyesters. Polymers that can be produced in this manner include but are not limited to for example, polyhydroxybutyrate, and copolymers incorporating both short chain length and medium chain length monomers, such as polyhydroxybutyrate -co- polyhydroxycaproate, polyhydroxycaproate -co- polyhydroxyoctanoate, and polyhydroxyoctanoate -co- polyhydroxydecanoate.

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to

the compositions and/or methods and in the steps or in the sequence of steps of the methods described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention.

5

**WHAT IS CLAIMED IS:**

1. A nucleic acid segment encoding:
  - 5 a) a polyhydroxyalkanoic acid synthase protein; and
  - b) a fatty acid:acyl-CoA transferase protein.
2. The nucleic acid segment of claim 1, wherein the polyhydroxyalkanoic acid synthase protein is an *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein.
- 10 3. The nucleic acid segment of claim 2, wherein the *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein is encoded by the *Alcaligenes eutrophus phaC* polyhydroxyalkanoic acid synthase structural gene.
- 15 4. The nucleic acid segment of claim 1, wherein the fatty acid:acyl-CoA transferase protein is a 4-hydroxybutyrate:acyl-CoA transferase protein.
5. The nucleic acid segment of claim 4, wherein the 4-hydroxybutyrate:acyl-CoA transferase protein is a *Clostridium kluyveri* 4-hydroxybutyrate:acyl-CoA transferase protein.
- 20 6. The nucleic acid segment of claim 5, wherein the *Clostridium kluyveri* 4-hydroxybutyrate:acyl-CoA transferase protein is encoded by the *Clostridium kluyveri orfZ* 4-hydroxybutyrate:acyl-CoA transferase structural gene.
- 25 7. The nucleic acid segment of claim 1, further comprising a promoter functional in bacterial cells.
8. The nucleic acid segment of claim 1, wherein:
  - 30 a) the sequence encoding a polyhydroxyalkanoic acid synthase protein is operably linked to its native promoter; and

- b) the sequence encoding a fatty acid:acyl-CoA transferase protein is operably linked to its native promoter.
9. The nucleic acid segment of claim 1, further comprising a promoter heterologous to:
- 5 a) the sequence encoding a polyhydroxyalkanoic acid synthase protein; and  
b) the sequence encoding a fatty acid:acyl-CoA transferase protein.
10. A recombinant vector comprising the nucleic acid segment of claim 1.
- 10 11. A recombinant vector comprising the nucleic acid segment of claim 9.
12. The recombinant vector of claim 11, further defined as vector pKSSE5.3.
13. The recombinant vector of claim 11, further defined as vector pSKSE5.3.
- 15 14. A recombinant vector comprising:
- a) a nucleic acid segment encoding a polyhydroxyalkanoic acid synthase protein;  
b) a nucleic acid segment encoding a fatty acid:acyl-CoA transferase protein; and  
c) a promoter heterologous to the nucleic acid segment encoding a  
20 polyhydroxyalkanoic acid synthase protein, and heterologous to the nucleic acid segment encoding a fatty acid:acyl-CoA transferase protein.
15. A cell comprising a nucleic acid segment, the nucleic acid segment encoding:
- a) a polyhydroxyalkanoic acid synthase protein; and  
25 b) a fatty acid:acyl-CoA transferase protein.
16. The cell of claim 15, further defined as a plant cell, mammalian cell, insect cell, fungal cell, or bacterial cell.
- 30 17. The cell of claim 16, wherein the cell is a plant cell.

18. The cell of claim 16, wherein the cell is a bacterial cell.
19. The bacterial cell of claim 18, wherein the bacterial cell is *Escherichia coli*.
- 5 20. The bacterial cell of claim 19, wherein the bacterial cell is *Escherichia coli* strain XL1-Blue.
21. The cell of claim 15, wherein the polyhydroxyalkanoic acid synthase protein is a *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein.
- 10 22. The cell of claim 21, wherein the *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein is encoded by the *Alcaligenes eutrophus phaC* polyhydroxyalkanoic acid synthase structural gene.
- 15 23. The cell of claim 15, wherein the fatty acid:acyl-CoA transferase protein is a 4-hydroxybutyrate:acyl-CoA transferase protein.
24. The cell of claim 23, wherein the 4-hydroxybutyrate:acyl-CoA transferase protein is a *Clostridium kluyveri* 4-hydroxybutyrate:acyl-CoA transferase protein.
- 20 25. The cell of claim 24, wherein the *Clostridium kluyveri* 4-hydroxybutyrate:acyl-CoA transferase protein is encoded by the *Clostridium kluyveri orfZ* 4-hydroxybutyrate:acyl-CoA transferase structural gene.
- 25 26. A method for preparing a transformed cell, comprising the steps:
- a) selecting a host cell;
  - b) contacting the host cell and a nucleic acid segment, the nucleic acid segment encoding:
    - i) a polyhydroxyalkanoic acid synthase protein; and
    - 30 ii) a fatty acid:acyl-CoA transferase protein;

under conditions suitable for uptake of the nucleic acid segment by the host cell; and

c) regenerating the cell to produce a transformed cell.

- 5 27. The method of claim 26, wherein the contacting step is further defined as calcium chloride mediated transformation.
28. The method of claim 26, wherein the cell is a plant cell, mammalian cell, insect cell, fungal cell, or bacterial cell.
- 10 29. The method of claim 28, wherein the cell is a plant cell.
30. The method of claim 28, wherein the cell is a bacterial cell.
- 15 31. The method of claim 30, wherein the bacterial cell is *Escherichia coli*.
32. The method of claim 31, wherein the bacterial cell is *Escherichia coli* strain XL1-Blue.
- 20 33. The method of claim 26, wherein the polyhydroxyalkanoic acid synthase protein is an *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein.
34. The method of claim 33, wherein the *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein is encoded by the *Alcaligenes eutrophus phaC* polyhydroxyalkanoic acid synthase structural gene.
- 25 35. The method of claim 26, wherein the fatty acid:acyl-CoA transferase protein is a 4-hydroxybutyrate:acyl-CoA transferase protein.
- 30 36. The method of claim 35, wherein the 4-hydroxybutyrate:acyl-CoA transferase protein is a *Clostridium kluyveri* 4-hydroxybutyrate:acyl-CoA transferase protein.



37. The method of claim 36, wherein the *Clostridium kluyveri* 4-hydroxybutyrate:acyl-CoA transferase protein is encoded by the *Clostridium kluyveri* orfZ 4-hydroxybutyrate:acyl-CoA transferase structural gene.
- 5 38. A method for the preparation of a polyester, comprising the steps of:
- a) obtaining a cell capable of producing:
    - i) a polyhydroxyalkanoic acid synthase protein; and
    - ii) a fatty acid:acyl-CoA transferase protein;
  - b) establishing a culture of the cell;
  - 10 c) culturing the cell under conditions suitable for the production of the polyester; and
  - d) isolating the polyester from the cell.
39. The method of claim 38, wherein the cell is a plant cell, mammalian cell, insect cell, fungal cell, or bacterial cell.
- 15 40. The method of claim 39, wherein the cell is a plant cell.
41. The method of claim 39, wherein the cell is a bacterial cell.
- 20 42. The method of claim 41, wherein the bacterial cell is *Escherichia coli*.
43. The method of claim 42, wherein the bacterial cell is *Escherichia coli* strain XL1-Blue.
- 25 44. The method of claim 38, wherein the polyhydroxyalkanoic acid synthase protein is a polyhydroxyalkanoic acid synthase protein from *Alcaligenes eutrophus*.
45. The method of claim 44, wherein the *Alcaligenes eutrophus* polyhydroxyalkanoic acid synthase protein is encoded by the *Alcaligenes eutrophus* phaC polyhydroxyalkanoic acid synthase structural gene.
- 30

46. The method of claim 38, wherein the fatty acid:acyl-CoA transferase protein is a 4-hydroxybutyrate:acyl-CoA transferase protein.
47. The method of claim 46, wherein the 4-hydroxybutyrate:acyl-CoA transferase protein is a *Clostridium kluyveri* 4-hydroxybutyrate:acyl-CoA transferase protein.
48. The method of claim 47, wherein the *Clostridium kluyveri* 4-hydroxybutyrate:acyl-CoA transferase protein is encoded by the *Clostridium kluyveri* orfZ 4-hydroxybutyrate:acyl-CoA transferase structural gene.
49. The method of claim 38, wherein the culture contains glucose.
50. The method of claim 38, wherein the culture contains 4-hydroxybutyric acid, the sodium salt of 4-hydroxybutyric acid,  $\gamma$ -butyrolactone, 1,4-butanediol, 4-hydroxyvaleric acid,  $\gamma$ -valerolactone, 1,4-pentanediol, 3-hydroxybutyric acid, the sodium salt of 3-hydroxybutyric acid, a hydroxypropionic acid, a hydroxybutyric acid, a hydroxyvaleric acid, a hydroxycaproic acid, a hydroxyheptanoic acid, a hydroxyoctanoic acid, a hydroxydecanoic acid,  $\gamma$ -caprolactone,  $\gamma$ -heptanolactone,  $\gamma$ -octanolactone, or  $\gamma$ -decanolactone.
51. The method of claim 38, wherein the culture contains molecular oxygen.
52. The method of claim 38, wherein the cell is further capable of producing a protein capable of hydrolysing a lactone to the corresponding hydroxyalkanoic acid.
53. The method of claim 38, wherein the cell is further capable of producing a 2-oxoglutarate decarboxylase protein and a 4-hydroxybutyrate dehydrogenase protein.
54. The method of claim 38, wherein the cell is further capable of producing a succinate:acetyl-CoA transferase protein, a succinate-semialdehyde dehydrogenase protein, and a 4-hydroxybutyrate dehydrogenase protein.

55. The method of claim 38, wherein the cell is further capable of producing a succinate-semialdehyde dehydrogenase protein, and a 4-hydroxybutyrate dehydrogenase protein.
- 5 56. The method of claim 38, wherein the cell is further capable of producing a 2-methylcitrate synthase protein, a 2-methylcitrate dehydratase protein, a 2-methylisocitrate dehydratase protein, a 2-methylisocitrate lyase protein, a succinate:acetyl-CoA transferase protein, a succinate-semialdehyde dehydrogenase protein, and a 4-hydroxybutyrate dehydrogenase protein.
- 10 57. The method of claim 38, wherein the polyester is a homopolyester.
58. The method of claim 57, wherein the homopolyester is poly(4-hydroxybutyric acid).
- 15 59. The method of claim 57, wherein the homopolyester is poly(3-hydroxybutyric acid).
60. The method of claim 38, wherein the polyester is a copolyester.
61. The method of claim 60, wherein the copolyester is poly(3-hydroxybutyric acid -co- 4-hydroxybutyric acid).
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# INTERNATIONAL SEARCH REPORT

Inter national Application No  
PCT/US 97/03994

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/52 C12N15/54 C12N15/70 C12N1/21 C12P7/62  
C12N15/82 C12N5/14 //(C12N1/21,C12R1:19)

According to International Patent Classification(IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 518 907 A (DENNIS DOUGLAS E) 21 May 1996 see claims ---	1
A	WO 95 21257 A (INNOVATIVE TECH CENTER) 10 August 1995 see claims ---	1
A	WO 93 06225 A (INNOVATIVE TECH CENTER) 1 April 1993 see claims --- -/--	1

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

19 November 1997

Date of mailing of the international search report

04/12/1997

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# INTERNATIONAL SEARCH REPORT

Inter      nal Application No  
PCT/US 97/03994

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	<p>CHEMICAL ABSTRACTS, vol. 127, Columbus, Ohio, US; abstract no. 219607, HEIN, SILKE ET AL: "Biosynthesis of poly(4-hydroxybutyric acid) by recombinant strains of Escherichia coli" XP002047459 see abstract &amp; FEMS MICROBIOL. LETT. (1997), 153(2), 411-418 CODEN: FMLED7;ISSN: 0378-1097, -----</p>	1-61

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/03994

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US 5518907 A	21-05-96	US 5371002 A	06-12-94
WO 9521257 A	10-08-95	EP 0742828 A	20-11-96
WO 9306225 A	01-04-93	US 5371002 A	06-12-94
		AU 2147492 A	27-04-93
		EP 0630410 A	28-12-94
		JP 6510425 T	24-11-94
		US 5569595 A	29-10-96